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Attorney Docket No. P50836

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Applicant:	John White	22 July, 2002	
Serial No.:	09/786,839	Group Art Unit:	1614
Filed:	March 1, 2001	Examiner:	Delacroix-Muirhei, C.
For:	CXCR2 INHIBITORS AND PMN ADHESION AND T-CELL CHEMOTAXIS		

Assistant Commissioner for Patents
Washington, D.C. 20231

SECOND DECLARATION UNDER 37 C.F.R. § 1.131(A)

I, John R. White, do hereby declare:

1. That I am the inventor of the subject matter claimed in the above referenced application;

2. That prior to December 31, 1997, the publication date of the Widdowson reference (International Publication No. WO 97/49400), the invention claimed in the referenced application was conceived and reduced to practice as evidenced by the following:

a) Prior to December 31, 1997, SmithKline Beecham (predecessor company to GlaxoSmithKline) entered into a service agreement with Montana ImmunoTech Inc. to perform experiments designed by me to investigate the role of the IL-8B (a.k.a. CXCR2) receptor and its antagonism in neutrophil-endothelial cell adhesive interactions;

b) Prior to December 31, 1997, Dr. Robert F. Bargatze, Vice President & Senior Scientist at Montana Immunotech Inc. delivered to me a report on the above described investigation (a true copy of which is attached hereto as Exhibit A). The reported results confirmed my hypothesis that compounds that bind to the CXCR2 receptor inhibit or block the binding of human neutrophils to activated endothelial cells; and

c) Prior to December 31, 1997, Judy Lee, under my direction, performed the experiments set forth at pages 29904-96 to 29904-97 of her laboratory notebook (a true copy of which is attached hereto as Exhibit B) wherein it was shown that compounds that bind to the CXCR2 receptor inhibit IL-8- or Groα-mediated human T-cell chemotaxis in a dose dependent manner;

3. That each of the dates redacted from Exhibits A and B is before December 31, 1997; and

4. That all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

signed: ______________________ dated: 22 July 2002

John R. White

Exhibit A

Report:
Investigation of the role of the IL-8B receptor and its antagonism in neutrophil-endothelial cell adhesive interactions.

For:

Dr. John R. White
SmithKline Beecham plc
Dept Molecular Immunology
P.O. Box 1539
King of Prussia, PA 19406

Prepared by:	Robert F. Bargatze Ph.D.
	Vice President & Senior Scientist
	Montana ImmunoTech Inc.
	Bozeman, MT 59715
Phone:	(406) 585-2733
FAX:	(406) 585-2766
e-mail	robfb@imt.net

Project goals

The objective of this project has been to investigate, in vitro, the role of the IL-8BR in recruitment of neutrophils to 4hr IL-1ß activated HUVEC. A low nM range inhibitor specifically acting on IL-8BR will be employed to prevent IL-8BR function. This will provide a means of assessing the degree to which the IL-8BR participates in signaling dependent arrest of neutrophils on endothelial cells during flow induced shear.

The following tasks were completed

Analysis of neutrophil / 4hr activated HUVEC rolling and sticking under flow induced shear for examining the function of the neutrophil IL-8BR and the effects of the antagonist on the regulation of integrin/selectin dependent neutrophil adhesion.

Methods

Neutrophil isolation

Neutrophils were isolated from human peripheral blood. Briefly, blood was collected into citrate anti-coagulant tubes (Becton Dickinson), diluted 1:2 at room temperature in sterile HEPES (20mM) buffered HBSS (pH 7.0) (Fisher Scientific), underlayed with Histopaque 1077 and Histopaque 1117 (Sigma), and centrifuged at 2,300 RPM for 30 min at room temperature. neutrophils were collected from the Histopaque 1117/1077 interface.

Analysis of the effects the IL-8 or Gro_a on the regulation of selectin/integrin mediated neutrophil rolling and arrest on IL-8 preincubated, IL-1ß activated HUVEC during flow induced shear.

The analysis of IL-8 and controls were performed in triplicate utilizing HBSS buffer containing 1% human serum buffered with 20mM HEPES. Human neutrophils were isolated, washed and infused into the shear system loop for recirculation through the capillary tube lined with confluent cultures of HUVEC pretreated with IL-8 (100ng) for 3 hours (Expts 1 and 3) or 7 hours (Expt 2), activated for 1hr with IL-1β (10ng) followed by a 2-3 hr incubation. Groα (5nM, 3 hr preincubation) experiments were performed using the same method as for the IL-8 preincubation experiments.

Analysis of the effects of neutrophil pretreatment with the SmithKline IL-8BR antagonist on selectin/integrin mediated neutrophil rolling and arrest on IL-8 pretreated, IL-1β activated HUVEC during flow induced shear.

The analysis of IL-8BR antagonist were performed in triplicate in HBSS buffer

sticking. For preliminary experiments in the study neutrophils were introduced into the assay loop without HUVEC IL-8 pretreatment. In these experiments IL-8 (100ng/ml) was infused into the assay loop (or not infused) after neutrophil rolling was established on the HUVEC monolayer. Soluble IL-8 treatment during the course of these assays induced no increased sticking of neutrophils beyond that seen in the untreated controls (data not shown).

GRO_a induction of enhanced neutrophil sticking

GRO_a preincubation with HUVECS produced an effect similar to that of IL-8 for inducing neutrophil adhesion (figures 3,6). Accumulation was far above that seen for the non-GRO_a/IL-8 treated assays and shows that the CXCR2 receptor is involved in induction of enhanced neutrophil sticking during shear dependent recruitment.

SB 237844 inhibition of IL-8 enhanced neutrophil sticking

Data in three replicate experiments (figures 2, 5, 8) show that the SmithKline IL-8RB antagonist after a 10 minute preincubation with neutrophils effectively inhibits IL-8 enhanced adhesion to IL-1 activated HUVECs. One additional experiment has been performed were the same observation has been repeated but the data has not be quantified and graphed.

Flow cytometric analysis

Neutrophils showed IL-8 induced shedding of L-selectin indicating that the IL-8 was binding and triggering neutrophil activation. Interestingly, this treatment did not induce significant changes in the expression of LFA-1, MAC-1 expression as might be expected for increasing the potential for stable static neutrophil adhesion.

Surface expression of L-selectin and LFA-1/MAC-1 on 30 minute IL-8 incubated neutrophils as revealed by mean fluorescent levels

	Leu-8	Leu-15
Control	246	882
IL-8, 100ng	116	892
IL-8, 1.0ng	100	816
IL-8, 0.1ng	127	834

Conclusions

• The data collected thus far in the study suggest that the SmithKline IL-8RB antagonist SB237844 is a potent inhibitor of IL-8 induced neutrophil-endothelial sticking under physiological shear conditions.

• Surprisingly, IL-8 is not an effect inducer of rapid neutrophil adhesion when administered in a soluble form after neutrophil rolling was established in the shear assay. Flow cytometry analysis did show that the IL-8 in solution did actively induced L-selectin shedding, as expected, but did not induce the expected increased expression of MAC-1/LFA-1. This observation suggested that the soluble IL-8 may not be inducing sufficient neutrophil β_2 integrin supported adhesion to cause rolling neutrophils to slow and stick on the HUVEC surface.

• Importantly, the HUVEC-IL-8 preincubation data does show that IL-8 when preincubated with HUVECs, which are subsequently activated with IL-1β, is a potent inducer of rapid neutrophil-endothelial cell sticking that can be efficiently blocked by the SB237844 IL-8RB antagonist.

Reference:

1) Bargatze, R.F., S. Kurk, G. Watts, T.K. Kishimoto, C.A. Speer, and M. A. Jutila. 1994. In vivo and in vitro functional examination of a conserved epitope of L- and E-selectin crucial for leukocyte-endothelial cell interactions. J. Immunol. 152:5814.

Figure 1

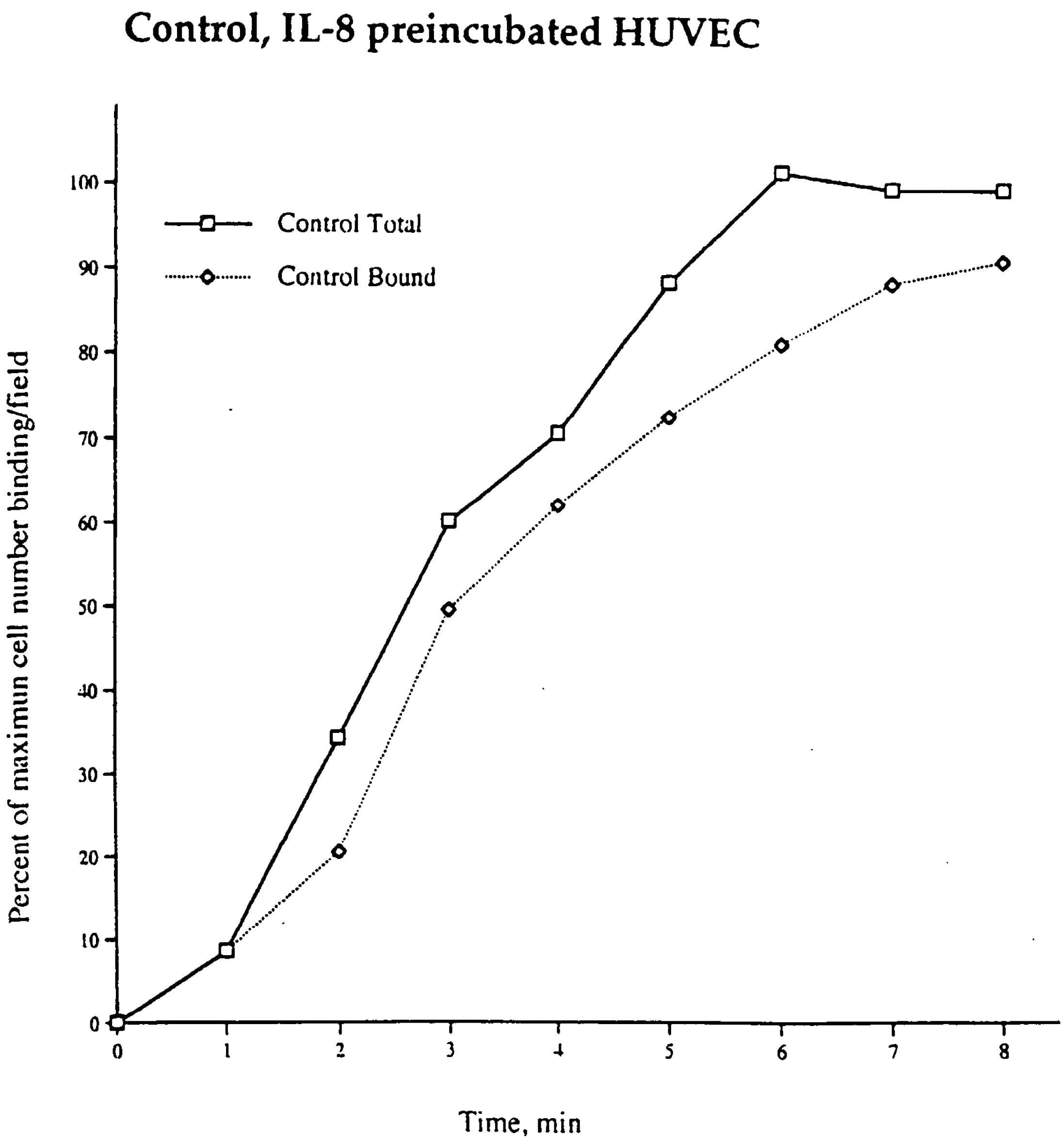

Control, IL-8 preincubated HUVEC
Control Total
Control Bound
Percent of maximun cell number binding/field
0
10
20
30
40
50
60
70
80
90
100
0
1
2
3
4
5
6
7
8
Time, min

Figure 2

SB 237844 treated neutrophils on IL-8 pretreated HUVEC

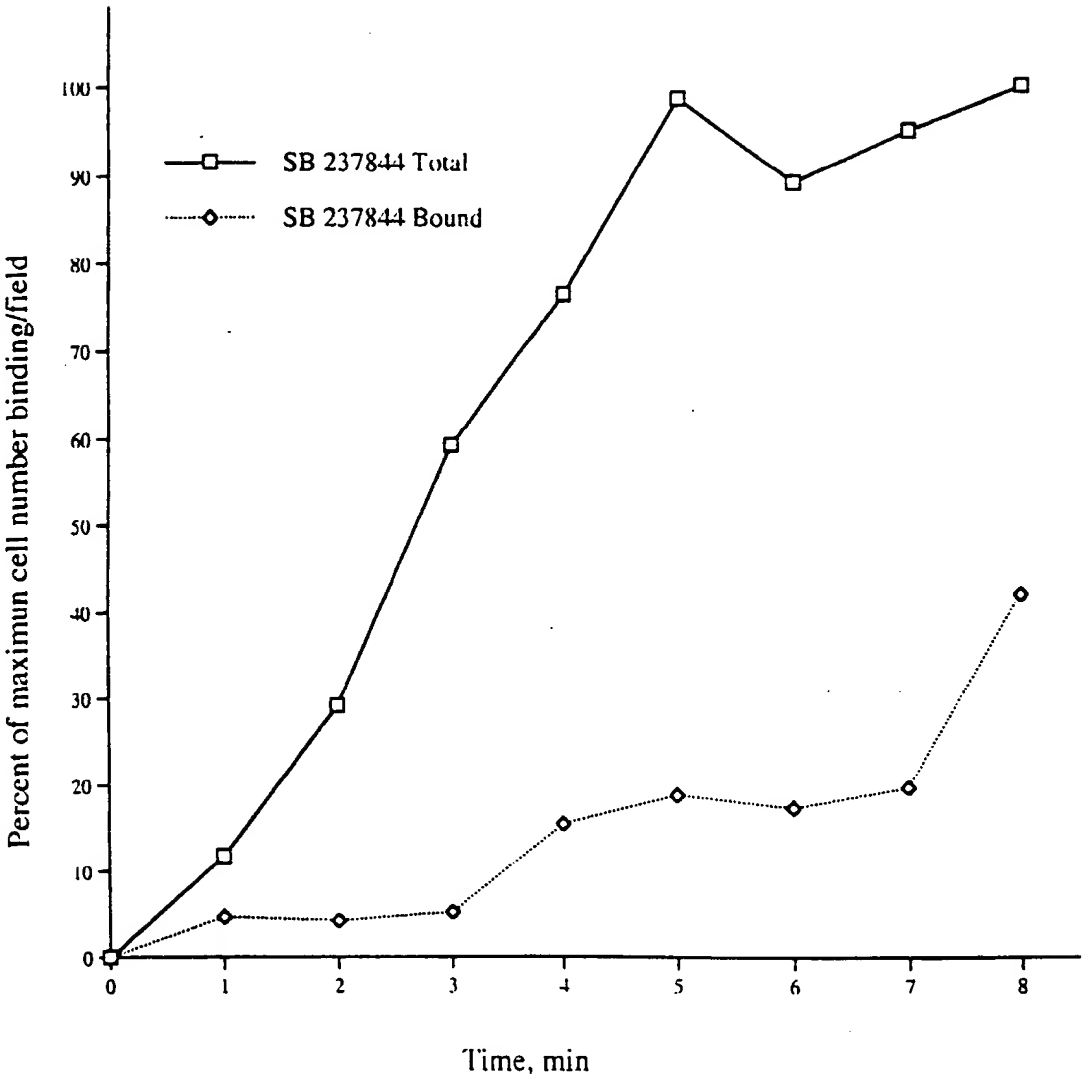


Figure 3

GROa treatment

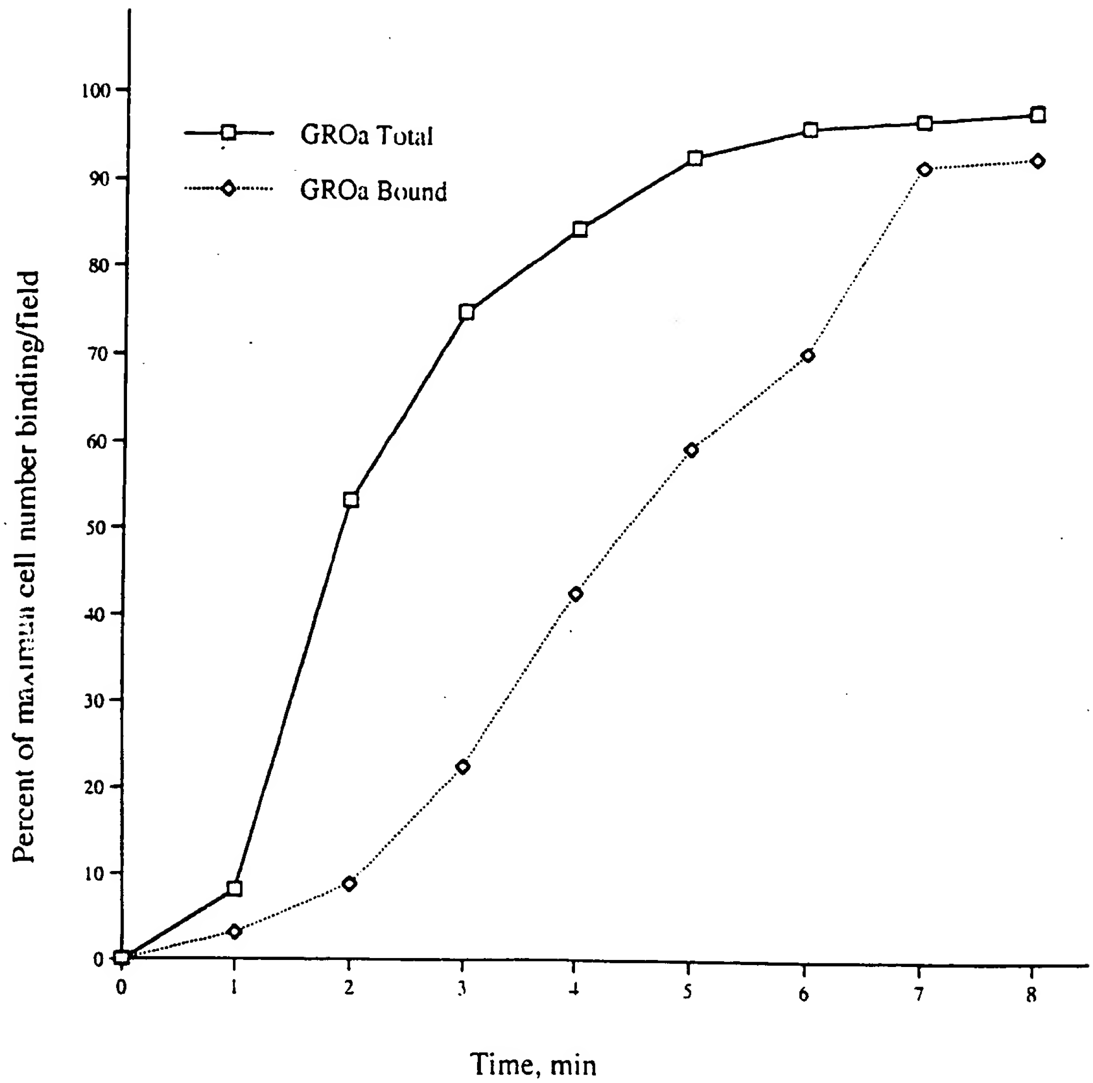
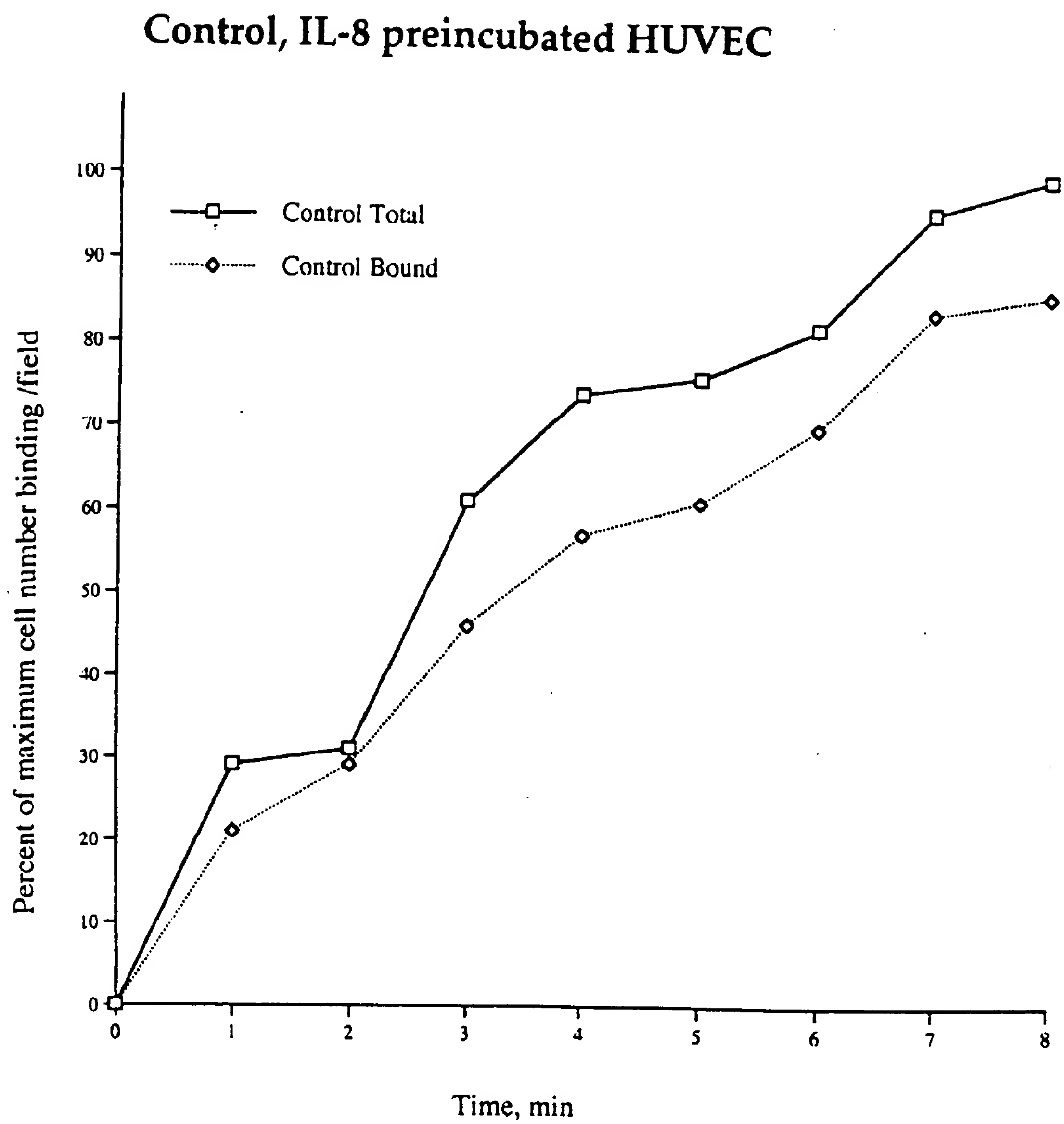


Figure 4



Control, IL-8 preincubated HUVEC
Control Total
Control Bound
Percent of maximum cell number binding /field
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
Time, min

Figure 5

SB 237844 treated neutrophils on IL-8 pretreated HUVEC

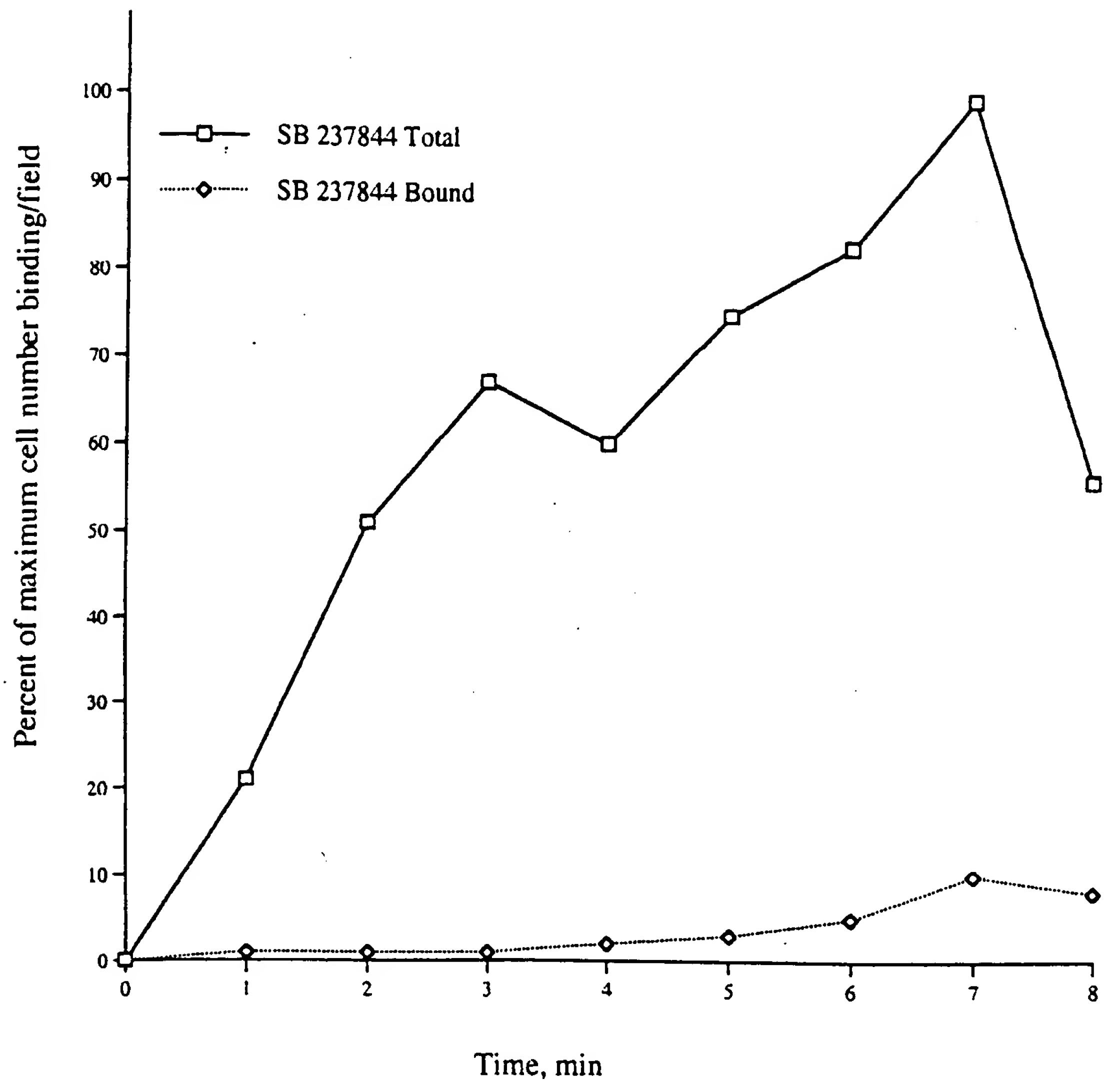


Figure 6

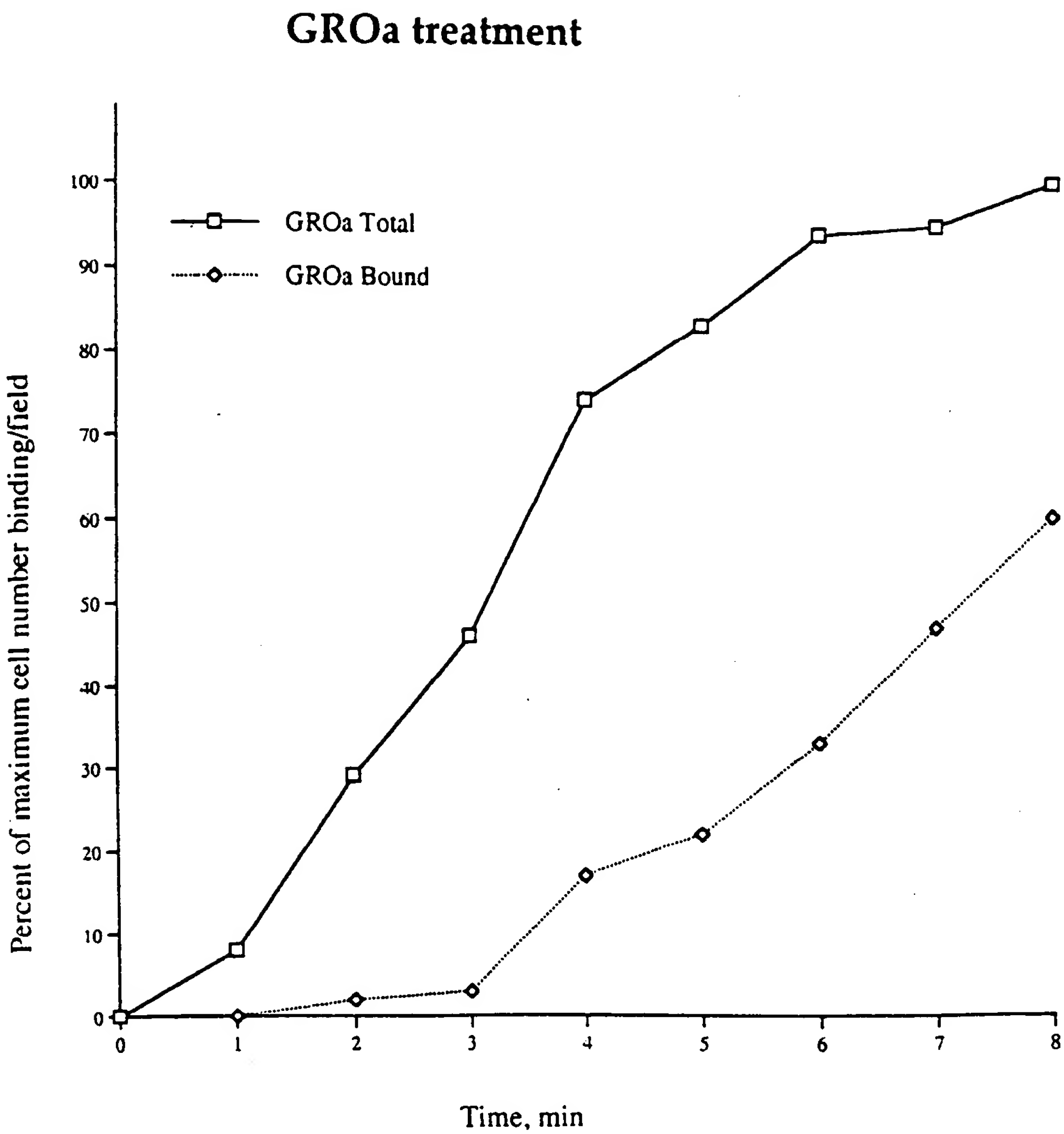
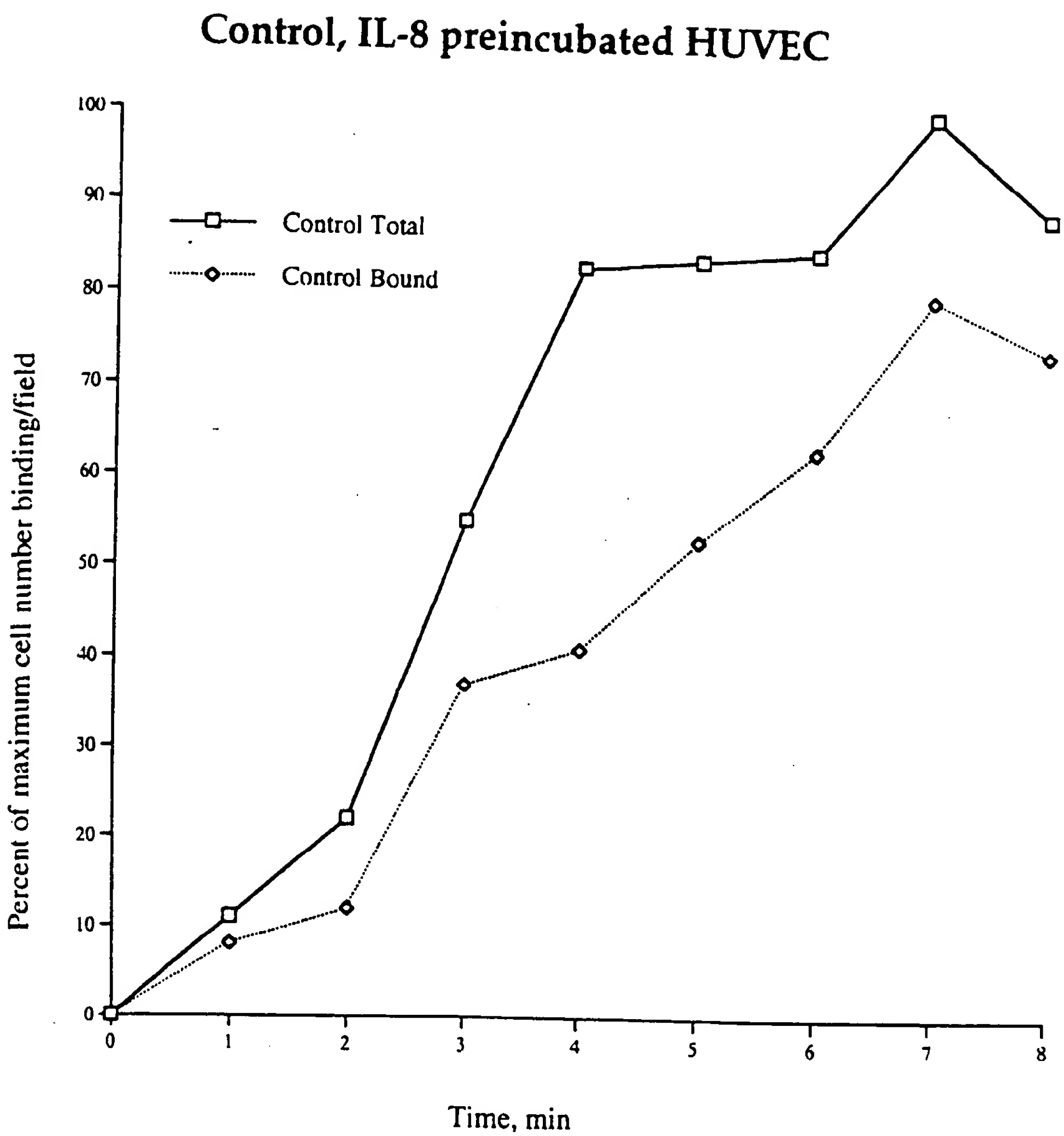

GROa treatment
GROa Total
GROa Bound
Percent of maximum cell number binding/field
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
Time, min

Figure 7



Control, IL-8 preincubated HUVEC
Control Total
Control Bound
Percent of maximum cell number binding/field
Time, min
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5
6
7
8

Figure 8

SB 237844 treated neutrophils on IL-8 pretreated HUVEC

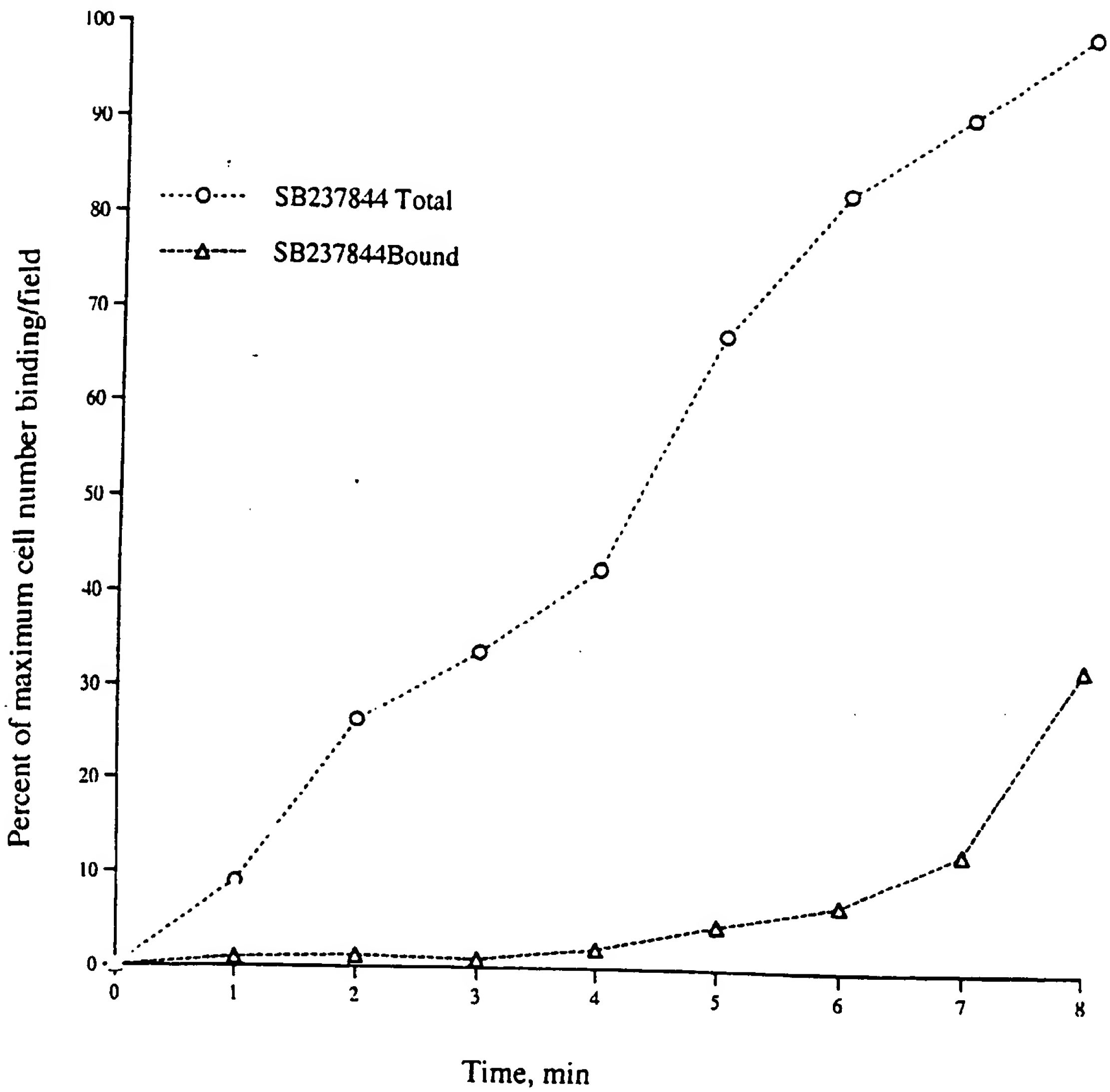


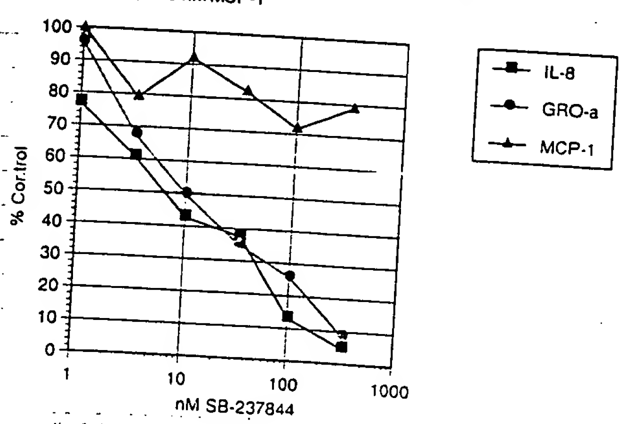
Exhibit B

T-cell migration; response affected by SB-237844

1. Donor; John White (immunized w/ tetanus booster)
   A. set up culture — 108 ml blood → 176×10^6 lymphocytes
   B. set up 2 × T-150 flasks w/ 20 ml each + 1:850 Tet. Toxo
2. After five day culture → 56×10^6 cells
   A. Use 5×10^6 cells to stain for CXCR1 vs CXCR2 for FACS
   B. For chemotaxis, need 8×10^6 cells
3. Agonists:
   5 nM IL-8, 15 nM GRO-α, 5 nM MCP-1
4. Buffer: RPMI + 0.5% ΔFCS
5. SB-237844 stock in 100% DMSO

Final	500 X	50% DMSO : 50% Buffer		Final
330 nM	165 µM	6 µl of 28 mM stock + 0.994 ml		
100 nM	50 µM	0.3 ml of 165 µM + 0.7 ml		
33 nM	16.5 µM	0.1 ml of 165 µM + 0.9 ml	2 µl →	0.998 ml buff ↓
10 nM	5 µM	0.1 ml of 50 µM + 0.9 ml		
3.3 nM	1.65 µM	0.1 ml of 16.5 µM + 0.9 ml		0.1% DMSO final
1 nM	0.5 µM	0.1 ml of 5 µM + 0.9 ml		in assay

Effect of SB-237844 on human T-cell migration in response to 5 nM IL-8, 15 nM GRO-a, or 5 nM MCP-1



IC50
6.5 nM
10 nM
> 330 nM

10089?JL.doc

97

Judy Lee

Effect of SB-237844 on human T-cell migration in response to 5 nM IL-8, 15 nM GRO-a, or 5 nM MCP-1

10/8/97 J.White donor; 5 day tetanus toxoid stimulation

				average	C.I.	% Control
nM SB-237844	IL-8	IL-8	IL-8	IL-8	IL-8	IL-8
1	261	248	256	255	5.80	76.73
3.3	197	213	222	211	4.79	60.61
10	178	155	151	161	3.67	42.67
33	135	138	169	147	3.35	37.58
100	81	61	98	80	1.82	13.09
330	49	57	65	57	1.30	4.73
5 nM IL-8	277	371	309	319	7.25	100
blank	33	46	52	44	0.99	

				average	C.I.	% Control
nM SB-237844	GRO-a	GRO-a	GRO-a	GRO-a	GRO-a	GRO-a
1	272	276	268	272	6.63	95.45
3.3	240	203	169	204	4.98	67.36
10	203	146	134	161	3.93	49.59
33	142	120	114	125	3.06	34.85
100	87	105	118	103	2.52	25.76
330	68	52	64	61	1.50	8.40
15 nM GRO-a	290	275	283	283	6.89	100.00
blank	48	49	26	41	1.00	

				average	C.I.	% Control
nM SB-237844	MCP-1	MCP-1	MCP-1	MCP-1	MCP-1	MCP-1
1	377	267	278	307	5.49	99.74
3.3	245	264	250	253	4.52	78.17
10	318	299	246	288	5.14	91.93
33	289	254	247	263	4.70	82.28
100	295	244	172	237	4.23	71.83
330	256	265	243	255	4.55	78.84
5 nM MCP-1	305	336	284	308	5.51	
blank	63	53	52	56	1.00	

* cells counted @ 400 x magnification

SCIENTIST SIGNATURE: Judy Lee

WITNESS SIGNATURE: